NEAR-INFRARED SURFACE-ENHANCED-RAMAN-SCATTERING (SERS) MEDIATED IDENTIFICATION OF SINGLE, OPTICALLY TRAPPED, BACTERIAL SPORES

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ABSTRACT

A novel methodology has been developed for the investigation of bacterial spores. Specifically, this method has been used to probe the spore coat composition of several *Bacillus* species. This technique may be useful in many applications; most notably, development of novel detection schemes toward potentially harmful biological agents. This method would also be useful as an ancillary environmental monitoring system where sterility is of importance (i.e., food preparation areas as well as invasive and minimally invasive medical applications). This unique detection scheme is based on the near-infrared (NIR) Surface-Enhanced-Raman-Scattering (SERS) from single, optically trapped, bacterial spores. The SERS spectra of several bacterial spores in aqueous media have been measured using SERS substrates based on 60-nm diameter gold colloids bound to 3-Aminopropyltriethoxysilane derivatized glass. The light from a 785-nm laser diode was used to capture/manipulate as well as simultaneously excite the SERS of an individual bacterial spore. The collected SERS spectra were examined for uniqueness and the applicability of this technique for the species identification of bacterial spores.

INTRODUCTION

Since the seminal work of Ashkin in 1970 based on a dual laser beam system[1] and later work in 1986 employing a single laser beam apparatus[2], the laser tweezer phenomenon which is based on using radiation pressure produced by tightly focusing a laser beam to capture and manipulate microscopic objects has been broadly accepted as a powerful tool to study viruses[3], vegetative bacterial cells[4-9] and colloidal crystallization in a microgravity environment[10]. More recently, this technique has been combined with varying Raman detection schemes to investigate inorganic gas bubbles[11], aerosols[12], the polymerization of emulsion particles[13], liquid-liquid molecular extraction processes of toluene in water[14], organic nanoparticles[15], vegetative cells[9] and solid-phase peptide synthesis[16]. Although significant work has been devoted to using this tool to study vegetative bacterial cells, to our knowledge there have been no reports in which optical trapping has been used to study bacterial spores. Further, optical trapping is ideally suited for the study of single spores since this technique has been used extensively in the study of micrometer sized dielectric particles.

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Raman spectroscopy has been an invaluable technique in the study of various chemical systems. The attractiveness of this technique stems from its narrow spectral band structure, lack of interference from water and relative insensitivity to the excitation wavelength employed. However, typically Raman spectral features are considered to be relatively weak thus requiring relatively lengthy collection times. In many circumstances, acquisition times can be shortened and spectral features can be improved by employing the Surface-Enhanced-Raman-Scattering (SERS) phenomenon.[17-19] Although the mechanism of the SERS effect is not fully understood, a plausible explanation for at least a portion of the enhancement has been attributed to an increase in the electromagnetic field encountered by the analyte. This electromagnetic field increase occurs when a metal surface is irradiated with the requisite wavelength of light and metal conduction electrons are excited to produce a surface plasmon resonance. Additionally, with certain metal-analyte systems enhancements from 4 to 14 orders magnitude are readily achieved. Since early reports on this technique in the late 1970's, its acceptance has not been as widespread as expected. This restricted acceptance is in part due to the lack of fabrication reproducibility in most SERS substrates. Relatively recent advances in SERS-active substrate fabrication have addressed this reproducibility issue. Further, these substrates are easily wavelength tunable, durable, biocompatible and possess a long shelf-life.[19]

We report here, to our knowledge, the first simultaneous exploitation of the optical trapping and Surface-Enhanced-Raman-Scattering phenomena. Further, a method based on their tandem application has been used to study seven *Bacillus* spores spanning three species; namely, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus thuringiensis*. In addition, we describe a near-infrared Raman Tweezer Detection System (NIR RTDS) that may be used to detect single, trapped particles (i.e., bacterial spores or polystyrene microspheres) as well as solutions. Preliminary Raman and SERS spectra measured using this method have been examined for uniqueness and the applicability of these spectra for the identification of bacterial spores at the species and strain levels.

MATERIALS AND METHODS

Instrumentation

The Raman tweezer detection system used in this study is similar to those described previously.[9,11-12,14-16] As illustrated in figure 1, 785-nm light from a laser diode was collimated through an interference filter (**IF**; Omega Optical; p/n 785DF10) with better than 70% transmission at 785-nm before passing through a Faraday optical isolator (**OI**; Electro-Optics Technology, Inc; Model # LD38I780). A 50-mm focal length lens (**FL**; Thor Labs, p/n LA1131-B) was placed after the optical isolator to expand the laser beam and effectively fill the back aperture of the microscope objective. The expanded beam was then deflected by a holographic SuperNotch-Plus™ filter (**SNF**; Kaiser Optical Systems, Inc.; p/n HSPF-785.0AR-1.0) centered at 785.0-nm into a Nikon TMS inverted microscope.

Raman scattering from a single, trapped particle was collected by the same 100X oil immersion objective and collimated onto and deflected by the dichroic mirror through two SNF's into a 0.275-m monochromator (SpectraPro®-275, Acton Research Corporation) and CCD (Spec-10:100BR, Roper Scientific) for spectral analysis. The monochromator was controlled and data was collected from the CCD by a personal computer using the WinSpec/32™ software package (Version 2.5.8.1, Roper Scientific). A home-built sample cell was constructed by drilling a 4.5-mm diameter hole in a 1.5-mm thick acrylic microscope slide. The sample well was sealed using a standard #1 glass cover slips. An area around the optical trap in the sample was imaged through the microscope objective and imaging optics (Edmund Industrial Optics) by a video camera (NEC TI-24A). A neutral density filter (**ND**) was placed before the imaging optics to further reduce the amount of laser light reaching the camera. Images from this video camera were displayed on a video monitor for observation and digitized for later inspection.

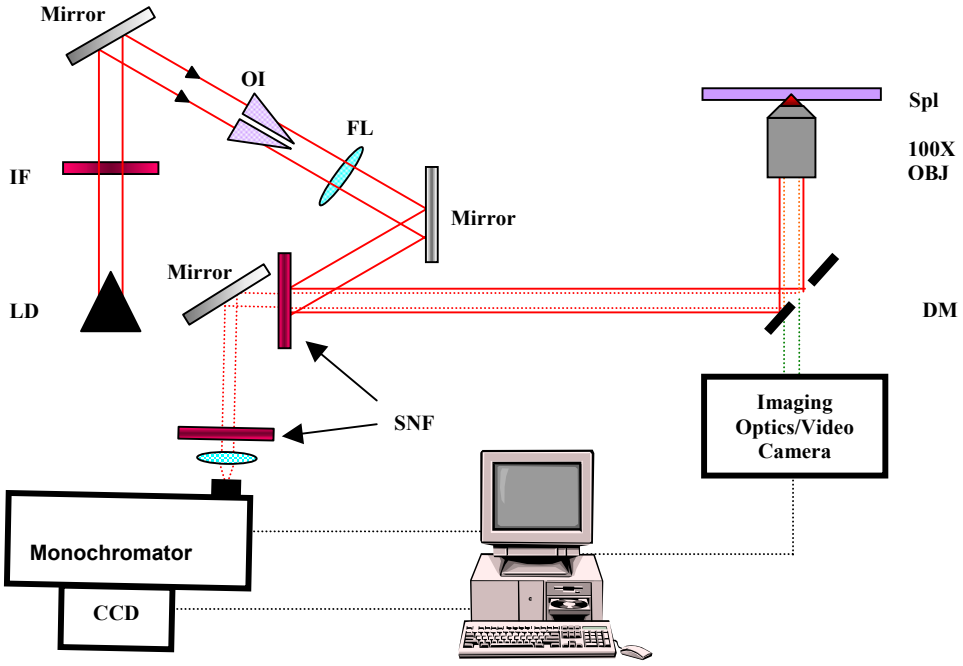

Figure 1. NIR Raman Tweezer Detection System. Laser Diode (**LD**), Interference Filter (**IF**), Faraday Optical Isolator (**OI**), Lens (**FL**), Dichroic Mirror (**DM**), Oil Immersion Objective (**100X OBJ**), Sample Cell (**Spl**), SuperNotch-Plus^{TM} filter (**SNF**) and Spec-10:100BR 1340 x 100 charge-couple-device (**CCD**). then focused through a 100X oil immersion microscope objective (**100X OBJ**; Nikon TE 300) into the sample. A dichroic mirror (**DM**) was placed inside of the microscope housing, before the sample to deflect the laser light into the microscope objective.

All spectra were collected with a laser excitation power of 20-mW (before the microscope), 15-ms integration, 45 co-additions at a spectral resolution of 7.5 cm^{-1} and are an average of three trials unless otherwise stated.

Chemicals and Bacterial Spores

Bacillus cereus (ATCC 11778, ATCC 12826 and ATCC 13824), *Bacillus megaterium* (ATCC 52) and *Bacillus thuringiensis* (ATCC 10792 and ATCC 35646) were received from the US Army Soldier Biological Chemical Command (SBCCOM; Aberdeen Proving Ground, MD). *Bacillus megaterium* (ATCC 8245) was purchased from Raven Biological Laboratories, Inc. (Omaha, NE). All spores were used as received.

3-Aminopropyltriethoxysilane (APTES) was purchased from the Sigma-Aldrich Chemical Company (Milwaukee, WI). 60-nm unconjugated gold colloids were received from Polysciences, Inc. (Warrington, PA). All chemicals were used as received.

SERS-substrate preparation

SERS-active substrates were prepared by a method similar to that suggested by Grabar, Freeman, Hommer and Natan[19] on 2- X 2-mm glass pieces.

RESULTS AND DISCUSSION

Normal Raman measurements

Bacillus cereus (Bc), *Bacillus megaterium* (Bm) and *Bacillus thuringiensis* (Bt), the three *Bacillus* spores selected for this study, were chosen for their taxonomic closeness to each other. In addition, the ubiquitous occurrence of bacterial spores makes their rapid determination of interest in various applications (namely, food preparation, medicine and agriculture). Further, the genetic similarity among Bc, Bm and Bt spores pose a challenge in their determination since they are expected to be similar in shape, size and immunoassay reactivity. Additionally, the spectroscopic investigation of individual spores is attractive since it: 1) is non-invasive; 2) is non-destructive; 3) facilitates the development of novel detection schemes toward potentially pathogenic biological agents; and 4) allows an estimation of the biodiversity among individual spores as well as spore species.

The performance of the Raman-Tweezer Detection System (RTDS) was estimated by collecting Raman spectra of both a solution and single trapped particle. Specifically, Raman spectra were collected of a saturated solution of Naphthalene ($C_{10}H_8$) in ethanol as well as a single trapped polystyrene microsphere suspended in water. These background corrected spectra (not shown) are rather clean and are in good agreement with published data. Further, the spectral features in both spectra are relatively narrow and show good a signal-to-noise (S/N) ratio. In addition, the polystyrene trapping experiment demonstrates it is possible to simultaneously trap and excite the Raman from a single 2-μm diameter particle suspended in deionized water using the RTDS. This is especially interesting in light that particles of this dimension approximate bacterial spores in size.

Background-corrected, intensity normalized Raman spectra of single, trapped *Bacillus cereus* (Bc, ATCC 12826), *Bacillus megaterium* (Bm, ATCC 52) and *Bacillus thuringiensis* (Bt, ATCC 35646) spores suspended in deionized water. The near identical spectra measured for Bc, Bm and Bt make it difficult to use these normal Raman spectra for their identification. However, it maybe possible to exploit the Surface-Enhanced-Raman-Scattering phenomenon to augment spectral features and facilitate their spectral discrimination.

Surface-Enhanced-Raman-Scattering (SERS) measurements

Samples were prepared for SERS measurements by placing the SERS-active substrate, 60-nm Au colloids immobilized on 2- X 2-mm glass, into the 4.5-mm diameter measurement well and adding a few hundred microliters of the dilute spore suspension. Subsequent to trapping, the captured spore was translated in close proximity to the SERS-active substrate and the SERS spectrum was collected. In addition, the spectral contribution due to the SERS substrate was estimated by blocking the laser beam until the spore wandered out of the trapping volume through Brownian motion. Once the spore was estimated to be outside the trapping volume the laser beam was unblocked and a background spectrum was collected.

Plotted in figure 2 is the SERS spectrum for a single, trapped Bacillus cereus (ATCC 13824) and the spectral contribution from the SERS-active substrate. As shown in the figure, most of the enhanced spectral features occur between 300 and 2040 cm^{-1} for the spore. These enhancements can be understood in light that the spore coat is primarily composed of high molecular weight proteins and lipids[20] that are expected to exhibit Raman spectral features in this region. More, since the surface-promoted

enhancement occurs over a relatively short range these features are most probably due to compounds contained solely in the spore coat. That is, this spectrum is attributed only to spore coat compounds. Further, the absence of these bands in the normal Raman spectra are most likely due to the low Raman scattering cross-section of most aliphatic organic molecules. That is, many unconjugated compounds and moieties do not, under normal circumstances, possess Raman-active modes. Additionally, it should be pointed out that the large background in this figure is similar in shape and position to that seen by other researchers[9] and most probably originates from one of the optical elements in the RTDS.

Shown in figure 3 are background-corrected, intensity normalized SERS spectra for several *Bacillus cereus* spore types; namely, ATCC 11778, ATCC 12826 and ATCC 13824. As illustrated in this figure, these Bc SERS spectra exhibit many spectral features that are not present in the normal Bc Raman spectrum. Further, these spectra are similar in shape with a maximum peak near 1600 cm^{-1}. Additionally, strong similarities are apparent in this figure between the ATCC 11778 and ATCC 12826 spectra. Specifically, both of these spectra reveal features at 1323, 1442, 2191, 2703 and 2950 cm^{-1}. However, a shoulder occurs at 1006 cm^{-1} in the ATCC 12826 spectrum that is not present in the ATCC 11778 spectrum. Conversely, the ATCC 13824 spectrum displays several relatively strong features between 300 and 1600 cm^{-1} in addition to bands at 1678 and 1996 cm^{-1}. These latter bands are especially interesting since they do not appear in either the ATCC 11778 or ATCC 12826 spectra. Additionally, it should be noted that the rather broad shape of these spectra is most likely due to closely spaced, unresolved bands

Plotted in figure 4 are background corrected, intensity normalized SERS spectra for *Bacillus megaterium* (ATCC 52) and *Bacillus megaterium* (ATCC 8245). Similarly to figure 3, these spectra are heavily overlapped with several spectral features that may be helpful in their discrimination. Specifically, bands occur at 678 and 1526 cm^{-1} in the Bm (ATCC 52) spectrum that are not present in the Bm (ATCC 8245) spectrum. In addition, shoulders are present in the ATCC 8245 spectrum at 1248 and 1315 cm^{-1} that are not observed in the ATCC 52 spectrum. Further, the band at 2171 cm^{-1} in the Bm ATCC 52 spectrum is slightly red-shifted to 2187 cm^{-1} in the Bm ATCC 8245 spectrum.

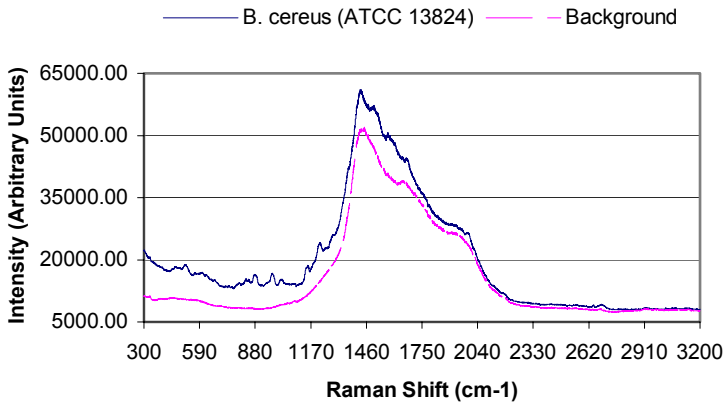

Figure 2: Single, trapped *Bacillus cereus* (ATCC 13824) spore SERS spectrum and spectral contribution due to SERS-active substrate. Taken with RTDS using 20-mW excitation, 15-ms integration and 45 co-addition.

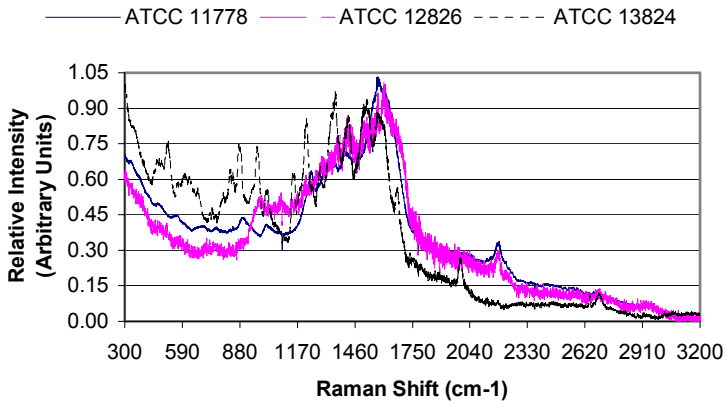

Figure 3: Single, trapped *Bacillus cereus* (ATCC 11778), *Bacillus cereus* (ATCC 12826) and *Bacillus cereus* (ATCC 13824) spore SERS spectra. Taken with RTDS using 20-mW excitation, 15-ms integration and 45 co-addition.

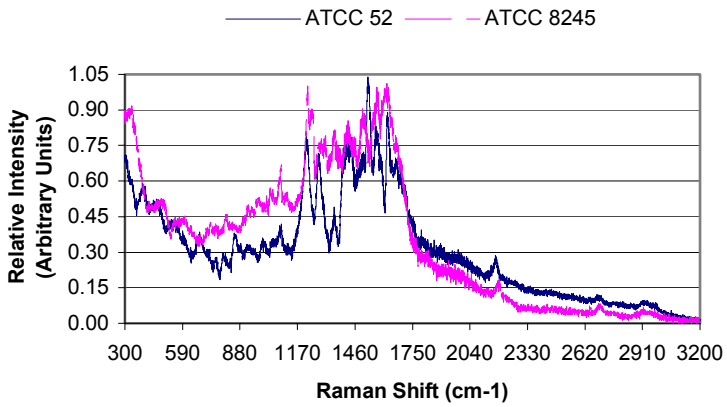

Figure 4: Single, trapped *Bacillus megterium* (ATCC 52) and *Bacillus megaterium* (ATCC 8245) spore SERS spectra. Taken with RTDS using 20-mW excitation, 15-ms integration and 45 co-addition.

Figures 3, 4 and 5 show that the observed spectral features make it possible to differentiate between single, optically trapped Bc (ATCC 11778), Bc (ATCC 12826) and Bc (ATCC 13824); Bm (ATCC 52) and Bm (ATCC 8245); and Bt (ATCC 10792) and Bt (ATCC 35646), respectively. Further, these figures taken together imply it is possible to use the SERS phenomenon to distinguish among single, optically trapped *Bacillus cereus*, *Bacillus megaterium* and *Bacillus thuringiensis*. That is, these data infer that the tandem exploitation of the SERS and optically trapping phenomena makes it possible to discriminate among the seven measured Bacillus spores (at the strain level); namely, *Bacillus cereus* (ATCC 11778), *Bacillus cereus* (ATCC 12826), *Bacillus cereus* (ATCC 13824), *Bacillus megaterium* (ATCC 52), *Bacillus megaterium* (ATCC 8245), *Bacillus thuringiensis* (ATCC 10792) and *Bacillus thuringiensis* (ATCC 35646).

Depicted in figure 5 are background corrected, intensity normalized SERS spectra for *Bacillus thuringiensis* (ATCC 10792) and *Bacillus thuringiensis* (ATCC 35646). As shown in this figure, these spectra may be differentiated by several features. For example, the Bt (ATCC 10792) spectrum is characterized by a broad set of peaks centered near 1340 cm^{-1}. Further, this spectrum contains obvious bands at 1134, 1180 and 1523 cm^{-1}. In contrast, the Bt (ATCC 35646) spectrum is composed of features

at 942, 1060, 1651 and 2194 cm-1 as well as a plateau at 1327 cm^{-1}. These features make it possible to distinguish between the Bt (ATCC 10792) and Bt (ATCC 35646) spectra.

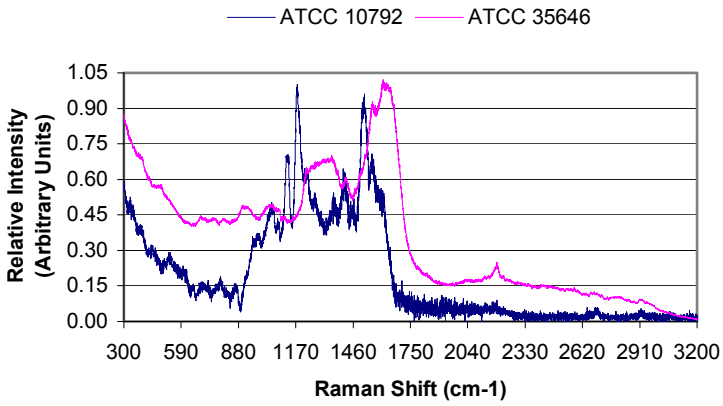

Figure 5: Single, trapped *Bacillus thuringiensis* (ATCC 10792) and *Bacillus thuringiensis* (ATCC 35646) spore SERS spectra. Taken with RTDS using 20-mW excitation, 15-ms integration and 45 co-addition.

CONCLUSIONS

A methodology is under development based on the simultaneous exploitation of the SERS and optically trapping phenomena. Preliminary data collected for seven Bacillus spores with a near-infrared Raman Tweezer Detection System (NIR RTDS) show it is difficult (if not impossible), to distinguish among these spores, using their normal Raman spectra; however, their SERS spectra imply that it is possible to discriminate among these *Bacillus* spores at the strain level. The measured complex spectral features make it difficult to uniquely identify any of the compounds contained in the spore coat. Further, using the NIR RTDS it is possible to acquire reasonable SERS spectra at a spectral resolution of 7.5 cm-1 with relatively meager trapping/excitation powers (20-mW) and short integration times (15-ms).

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REFERENCES

1. A. Ashkin, *Physical Review Letters*, **24**, 156 – 159, 1970.
2. A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, *Optics Letters*, **11**, 288 – 290, 1986.
3. A. Ashkin and J. M. Dziedzic, *Science*, **235**, 1517 – 1520, 1987.
4. A. Ashkin, J. M. Dziedzic, and T. Yamane, *Nature*, **320**, 769 – 771, 1987.
5. A. Ashkin, K. Schutze, J. M. Dziedzic, U. Euteneuer and M. Schliwa, *Nature*, **348**, 346 – 348, 1990.
6. W. H. Wright, G. J. Sonek, Y. Tadir, and M. W. Berns, *IEEE Journal of Quantum Electronics*, **26**, 2148 – 2157, 1990.

7. M. Ericsson, D. Hanstorp, P. Hagberg, J. Enger, and T. Nystrom , *Journal of Bacteriology*, **182**, 5551 – 5555, 2000.
8. J. Guck, R. Ananthakrishnan, H. Mahmood, T. J. Moon, C. C. Cunningham, and J. Kas, *Biophysical Journal*, **81**, 767 – 784, 2001.
9. C. Xie, M. A. Dinno, and Y. Li, *Optics Letters*, **27**, 249 – 251, 2002.
10. A. Resnick, *Review of Scientific Instruments*, **72**, 4059 – 4065, 2001.
11. M. Lankers, J. Popp, G. Rossling, and W. Kiefer, *Chemical Physics Letters*, **277**, 331 – 334, 1997.
12. J. F. Lubben, C. Mund, B. Schrader, and R. Zellner, *Journal of Molecular Structure*, **480 - 481**, 311 – 316, 1999.
13. V. E. Roman, J. Popp, E. Urlaub, S. Schlucker, M. Lankers, G. Robling, and W. Kiefer, *Journal of Molecular Structure*, **482 - 483**, 497 – 501, 1999.
14. K. Ajito, M. Morita, and K. Torimitsu, *Analytical Chemistry*, **72**, 4721 – 4725, 2000.
15. K. Ajito and K. Torimitsu, *Applied Spectroscopy*, **56**, 541 – 544, 2002.
16. M. P. Houlne, C. M. Sjorstrom, R. H. Uibel, J. A. Kleimeyer, and J. M. Harris, *Analytical Chemistry ASAP*, ac020325t, A – I, 2002.
17. J. R. Ferraro and K. Nakamoto, *Introductory Raman Spectroscopy*, Chapter 3, Academic Press, San Diego and London, 1994.
18. D. L. Gerrard, *Analytical Raman Spectroscopy*, J. G. Grasselli and B. J. Bulkin, 301 - 304, John Wiley and Sons, Inc., New York, 1991.
19. K. Grabar, R. G. Freeman, M. B. Hommer, and M. J. Natan, *Analytical Chemistry*, **67**, 735 – 743, 1995.
20. G. W. Gould and A. Hurst, *The Bacterial Spores*, Chapter 7, Academic Press, London and New York, 1969.